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Properties of *Manduca sexta* chitinase and its C-terminal deletions

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Abstract

Manduca sexta (tobacco hornworm) chitinase is a molting enzyme that contains several domains including a catalytic domain, a serine/threonine-rich region, and a C-terminal cysteine-rich domain. Previously we showed that this chitinase acts as a biopesticide in transgenic plants where it disrupts gut physiology. To delineate the role of these domains further and to identify and characterize some of the multiple forms produced in molting fluid and in transgenic plants, three different forms with variable lengths of C-terminal deletions were generated. Appropriately truncated forms of the *M. sexta* chitinase cDNA were generated, introduced into a baculovirus vector, and expressed in insect cells. Two of the truncated chitinases (Chi 1-407 and Chi 1-477) were secreted into the medium, whereas the one with the longest deletion (Chi 1-376) was retained inside the insect cells. The two larger truncated chitinases and the full-length enzyme (Chi 1-535) were purified and their properties were compared. Differences in carbohydrate compositions, pH–activity profiles, and kinetic constants were observed among the different forms of chitinases. All three of these chitinases had some affinity for chitin, and they also exhibited differences in their ability to hydrolyze colloidal chitin. The results support the hypothesis that multiple forms of this enzyme occur in vivo due to proteolytic processing at the C-terminal end and differential glycosylation. Published by Elsevier Science Ltd.

Keywords: Chitinase; Tobacco hornworm; C-terminal deletions; Kinetic properties; Chitin binding; Colloidal chitin digestion; Molting enzyme; Physical properties; *Manduca sexta*; Insect

1. Introduction

Chitinases have been isolated and characterized from a variety of sources including bacteria, fungi, plants, marine organisms, insects, and mammals (Koga et al., 1999; Fukamizo, 2000). They belong to two major families of glycosyl hydrolases (families 18 and 19), which differ in their amino acid sequences and their catalytic mechanisms (Henrissat and Bairoch, 1993; Coutinho and Henrissat, 1999). Even within the same family, members exhibit sequence diversity and variations in enzymatic properties. Perhaps the most remarkable difference among chitinases from different kingdoms is the large range in their sizes. The smallest plant chitinases are about 22 kDa (Neuhas, 1999), whereas the largest yeast chitinases have a size of about 130 kDa (Kuranda and Robbins, 1991). While glycosylation accounts for much of these differences, the length of the protein also varies among chitinases from different sources and sometimes from the same organism. The smallest chitinases consist primarily of a catalytic domain, whereas the larger chitinases often are organized into multiple domains with distinct functions (Blaak et al., 1993; Morimoto et al., 1997; Kramer and Muthukrishnan, 1997; Shen and Jacobs-Lorena, 1998; Watanabe et al., 1993). The following domains have been identified among these chitinases: a catalytic domain, one or more chitin-binding domains which can be either N-terminal or C-terminal to the catalytic domain, one or more fibronectin type III domains, serine/threonine-rich domains which may have a role in glycosylation and protein turnover (Rechsteiner and Rogers, 1996), and other conserved domains of unknown function (Blaak et al., 1993; Watanabe et al., 1993). Chitinases also differ in their pH optima, catalytic parameters, affinity for chitin and stability. Presumably, variations in their domain structure and amino acid sequences contribute to many of these differences.

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For several years, we have been interested in enhancing the enzymatic and pesticidal activities of chitinolytic enzymes (Kramer and Muthukrishnan, 1997). During the course of our studies on a chitinase and its gene from an insect source, namely the tobacco hornworm (*Manduca sexta*), we determined that this enzyme, which is critical for molting, is organized into multiple domains including an N-terminal catalytic domain, a middle serine/threonine-rich domain, and a C-terminal cysteine-rich domain (Kramer and Muthukrishnan, 1997). In the molting fluid from which this enzyme was isolated, three different forms of this protein with sizes of 50, 62 and 85 kDa were detected (Koga et al., 1983). All three were enzymatically active and cross-reacted immunologically. The 85 kDa protein was found to be glycosylated (Gopalakrishnan et al., 1995).

When the cDNA for the full-length *M. sexta* chitinase was expressed in transgenic tobacco plants, most of the insect chitinase was in a C-terminally truncated form with a size of only 46 kDa, which was devoid of glycosylation (Wang et al., 1996; Ding et al., 1998). This 46 kDa chitinase was less active than the 85 kDa protein, but it still had an insecticidal activity against the merchant grain beetle, *Oryzaphilus mercator*. However, we were unable to carry out extensive characterization of this enzyme because of its limited availability.

In this study, we describe some properties of three truncated forms of *M. sexta* chitinase, which are missing specific sequences at the C-terminus. The C-terminal-deleted forms of *M. sexta* chitinase were generated by deleting the corresponding parts of the cDNA and expressing the truncated genes in insect cells in culture. The properties of these proteins were compared.

Note: Chi 1-376, Chi 1-407, Chi 1-477 and Chi 1-535 refer to *M. sexta* chitinases extending from the mature N-terminus to amino acids at positions 376, 407, 477 and 535, respectively.

2. Materials and methods

2.1. Construction of expression vectors

The following primers were utilized for PCR amplification of *M. sexta* chitinase coding regions to generate the three C-terminal-deleted proteins, Chi 1-376, Chi 1-407 and Chi 1-477, which are missing all of the sequences of the mature *M. sexta* chitinase beyond amino acid positions 376, 407 and 477, respectively.

The forward primer:
Primer 5′-GGAATTCAAGATGCGAGCG-3′ 19-mer
Reverse primers:
Primer 376: 5′-TCCTGCAGTTACATGTGCTTATGAAG-3′ 26-mer
Primer 407: 5′-CCTGCAGTTATTCTGAAGGAT-3′ 21-mer
Primer 477: 5′-TCCTGCAGTTATTCGCTACCATCGAC-3′ 26-mer

The forward primer contains an EcoRI site (GAATTC) and the translation start site of the *M. sexta* chitinase gene, whereas all three of the reverse primers, 376, 407 and 477, have Pst I sites (CTGCAG) and the complement of a translation termination codon (TTA) immediately following it.

2.2. Construction of truncated forms of M. sexta chitinase gene

PCR reactions were carried out in a final reaction volume of 50 μl using an *M. sexta* chitinase cDNA clone (#10, Kramer et al., 1993) as the template, with 0.5 μM of the forward primer, and one of the three reverse primers for the three reactions in the GeneAmp® PCR System 2400 (Perkin–Elmer) using a denaturation temperature of 95°C, an annealing temperature of 56°C, and an elongation temperature of 72°C for 25 cycles. The PCR products were recovered after 1% agarose gel electrophoresis and cloned into pGEM-T vector (Promega). Plasmid DNAs prepared from colonies containing the truncated *M. sexta* chitinase cDNA were digested with Pst I and Eco RI, and the recovered DNA fragments then were ligated to the baculovirus transfer plasmid, pVL1393, digested with Pst I and Eco RI. In the transfer plasmids, the genes are under the control of the viral polyhedrin promoter. These plasmids were utilized to obtain the recombinant baculovirus, AcMNPV containing the chitinase genes, by co-transfection with BaculoGold™ DNA (PharMingen, San Diego, CA), as described previously (Gopalakrishnan et al., 1995). Recombinant baculoviruses were plaque purified, amplified in the *Spodoptera frugiperda* cell line, SF21, to obtain high-titer virus, and used in expression studies.

2.3. Expression of Chi 1-535, Chi 1-477, Chi 1-407 and Chi 1-376 in baculovirus expression system

For protein expression, approximately 2×10^6 *Trichoplusia ni* Hi-5 cells were seeded into a 25 cm^2 cell culture flask in 5 ml EX-CELL 400 serum-free medium (JRH Biosciences, Lenexa, KS). The cells were infected by virus with a high titer (10^8 ml^{-1}) at a multiplicity of infection of 3–5. The proteins expressed in the supernatants and cells were analyzed by SDS-PAGE and Western blotting as described by Gopalakrishnan et al. (1995).

For protein purification, a scale-up of expression was employed using 125 cm^2 flasks and 25 ml of medium. Media from several flasks (100–200 ml) were pooled. The culture medium was collected 3–5 days after infec-

tion and centrifuged at 1000*g* for 30 min. The supernatants were dialyzed against 4 l of 20 mM Tris–HCl, pH 7.8 for 24 h at 4°C and used for subsequent purification of the secreted forms of chitinase.

2.4. Purification of secreted chitinases from the culture medium

The dialyzed medium was passed through a 1.5×20 cm column of Q-Sepharose equilibrated with Tris–HCl buffer (pH 7.5) at a flow rate of 1 ml/min. Then the column was washed with 100 ml of 25 mM Tris–HCl buffer (pH 7.8). A 0–0.5 M gradient of NaCl in 25 mM Tris–HCl (pH 7.8) was used for elution of Chi 1-407 and Chi 1-535, whereas a 0–0.7 M gradient of NaCl was used for elution of Chi 1-477. Fractions of 2 ml were collected and peak fractions were identified by measuring A_{280}, as well as analysis by SDS-PAGE and Western blotting. Peak protein fractions containing *M. sexta* chitinase were pooled.

The pooled fractions of Chi 1-407 from the Q-Sepharose column were concentrated in a Centriprep-30 (Amicon, Beverly, MA) to a volume of 1.5 ml and then applied to a 1.5×30 cm Biogel P-100 gel filtration column (Bio-Rad, Hercules, CA), which was equilibrated with 25 mM phosphate–citrate buffer (pH 7.5). The column was eluted with the same buffer at 0.3 ml/min, and 2 ml fractions were collected. Peak fractions were identified by A_{280}, and then further analyzed by SDS-PAGE.

2.5. Purification of Chi 1-477 by hydrophobic interaction chromatography

The fractions from the Q-Sepharose column that contained Chi 1-477 were pooled and mixed with saturated ammonium sulfate to a final concentration of 1.6 M. The sample was then applied to a prepacked methyl hydrophobic interaction column (methyl HIC, 5 ml, Bio-Rad), which was equilibrated at 1 ml/min with 1.6 M ammonium sulfate in 25 mM Tris–HCl, pH 7.5. The column was washed with starting buffer, and eluted at a rate of 2 ml/min with a decreasing gradient of ammonium sulfate (from 1.6 to 0 M) in 25 mM Tris–HCl, pH 7.8. Fractions of 2 ml were collected. Peak protein fractions were selected by A_{280}, and then analyzed by SDS-PAGE and Western blotting.

2.6. Protein concentration determination

Protein concentration was determined using the bicinchoninic acid assay (BCA) (Pierce, Rockford, IA). Bovine serum albumin was used as the standard protein.

2.7. Chitinase activity assay using CM-Chitin-RBV as substrate

Chitinase activity was assayed using carboxymethyl Remazol Brilliant Violet chitin (CM-Chitin-RBV) as the substrate (Loewe Biochemica, Munich, Germany). Each enzyme sample was diluted with water to 0.4 ml, and incubated with 0.2 ml of CM-Chitin-RBV (2 mg/ml) and 0.2 ml of 0.2 M phosphate citrate buffer (pH 7.5) at 37°C for 2 h. The reaction was stopped by addition of 0.2 ml of 2 N HCl. The sample was cooled in ice for 15 min and then centrifuged at 12,000 rpm for 5 min. The supernatant was collected and its A_{550} was determined.

2.8. pH profile

The effect of pH on chitinase activity was studied using 0.5 μg of chitinase and CM-Chitin-RBV as substrate at 37°C for 1 h. The concentration of all buffers was 0.2 M and they were prepared according to Dawson et al. (1969). Buffer compositions were sodium citrate for pH 4–4.5, sodium acetate for pH 4.5–6, sodium phosphate for pH 6.0–8.0, and glycine–NaOH for pH 9–10.5. This experiment was done in duplicate.

2.9. Temperature profile

The optimum temperatures for enzyme activity were investigated by incubating 0.8 ml reaction mixture, which contained 0.2 ml of 0.2 M citrate buffer pH 7.5, 0.2 ml CM-Chitin-RBV substrate and 0.4 ml enzyme (0.5 μg), over the range of 0–60°C. The absorbance at 550 nm was recorded. This experiment was done in triplicate.

2.10. Kinetic analysis of enzymes

2.10.1. MU-$(GlcNAc)_3$ as substrate

The kinetic assays were conducted according to Hollis et al. (1997) with some modifications. The assays were done by using 4-methylumbelliferyl β-*N*,*N*′,*N*″-triacetylchitotrioside [MU-$(GlcNAc)_3$] from Sigma. MU-$(GlcNAc)_3$ was dissolved in 0.1 M sodium phosphate buffer, pH 6.0. The 50 μl reaction contained 48.5 μl of substrate solution, ranging in concentration from 0 to 50 mM, and 1.5 μl of enzyme (0.1 μg). The reaction was carried out at 37°C for 10 min and stopped by adding 12.5 μl 2 N HCl. The reaction mixture was diluted with 0.15 M glycine–NaOH buffer (pH 10.5) to 2 ml for determining the free methylumbelliferone released by enzymatic hydrolysis. Fluorescence spectrophotometry (Model F-4010 Fluorescence Spectrophotometer, Hitachi, Ltd, Tokyo, Japan) was used to measure product formed with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. A standard curve of free methylumbelliferone was made and shown to be linear over the range of product measurement, which allowed for the conversion of fluorescence counts to nanomoles of methylumbelliferone released per minute.

2.10.2. CM-chitin-RBV as substrate

The kinetic assays using CM-Chitin-RBV were carried out according to Gopalakrishnan et al. (1995) with some modifications. The 0.8 ml reaction mixture contained 0.3 ml substrate (ranging in concentration from 0.025 to 0.75 mg/ml), 0.3 ml enzyme (0.5 μg) and 0.2 ml of 0.2 M phosphate buffer (pH 6). The reaction was carried out at 37°C for 1 h, then stopped by adding 0.2 ml of 2 N HCl, and cooled on ice for 15 min. The reaction mixture was centrifuged at 12,000*g* for 5 min and the supernatant was used for A_{550} determination.

2.10.3. Calculation of kinetic constants

Reaction velocity data as a function of substrate concentration were analyzed using the ordinary Michaelis–Menten model for the polymeric substrate chitin RBV and a competitive substrate inhibition model for the oligosaccharide substrate MU-$(GlcNAc)_3$. To obtain the kinetic constants, a nonlinear least-squares fitting of the data was conducted using Kaleidagraph software which includes the Levenberg–Marquardt algorithm.

2.11. N-terminal amino acid sequence determination

Proteins were separated using SDS-PAGE and transferred onto a PVDF membrane, which was then stained with Coomassie Brilliant Blue. The protein band was cut out and subjected to sequence analysis by automated Edman degradation using an Applied Biosystems Sequencer at the Biotechnology Core Facility, Kansas State University, Manhattan, Kansas.

2.12. Molecular weight determination

Approximately 5 μg of Chi 1-407 and Chi 1-477 were used for molecular mass determination by laser desorption mass spectrometry at the Biotechnology Core Facility, Kansas State University, Manhattan, Kansas.

2.13. Chitin-binding assay

The chitin used for the binding assay was prepared according to Kuranda and Robbins (1991) and Venegas et al. (1996). Purified chitin (0.2 g) was boiled for 5 min in 5 ml 1% SDS and 1% β-mercaptoethanol. Then the 5 ml mixture was washed with 80 ml H_2O and filtered through a 0.45 μm filter. The retentate was resuspended in 2 ml of 25 mM sodium phosphate (pH 6). The binding assay was performed by mixing 100 μl of the chitin suspension with 200 μl of protein (3 μg) in a microcentrifuge tube. Then 50 μl of the protein–chitin mixture was removed and stored as starting fraction. This mixture was incubated with end over end rotation for 1 h at room temperature and then centrifuged for 10 min. The supernatant was removed and saved as the unbound fraction. Then the pellet was washed two times by resuspension in 75 μl of sodium phosphate buffer (pH 6), followed by centrifugation. Both supernatants were saved, and pooled as the wash fraction. The pellet was resuspended in 150 μl of 1×SDS-PAGE sample buffer and boiled for 5 min. After centrifugation, the supernatant was removed and saved as the bound fraction. All samples were subsequently analyzed by SDS-PAGE followed by staining with Coomassie Blue. A second treatment with the same elution buffer did not release additional protein.

2.14. Colloidal chitin digestion assay

Enzyme assays for full-length and C-terminal-deleted forms of chitinase were carried out using 0.4% colloidal chitin in agar as the substrate. Ten microliter aliquots containing 20 μg of each protein was applied to a Whatmann 3MM filter paper disk and incubated on a 1.5% agar plate containing colloidal chitin at 37°C for 16 h. Then, the chitin–agar plates were stained with 0.01% fluorescent brightener 28, viewed under UV light, and photographed using a green filter.

2.15. Carbohydrate composition of M. sexta *chitinases*

The protein samples purified by Q-Sepharose column chromatography followed by Biogel P-100 column gel filtration chromatography were desalted using a Centriprep-30 filter (Amicon, Beverley, MA). After hydrolysis, the samples were analyzed for monosaccharide composition by preparing the trimethylsilyl derivatives of the methyl glycosides followed by gas chromatography (GC-FID) and combined gas chromatography/mass spectrometry analysis at the Complex Carbohydrate Research Center, Athens, GA (http://www.ccrc.uga.edu).

3. Results

3.1. Effect of C-terminal truncation on signal peptide processing and secretion

Three constructs of the *M. sexta* chitinase gene, which had deletions corresponding to the regions coding for C-terminal amino acids beyond position 376 (Chi 1-376), 407 (Chi 1-407), and 477 (Chi 1-477) were prepared, introduced into *Autographa californica* nuclear polyhedrosis virus (AcMNPV) under the control of the polyhedrin promoter and expressed in insect cells along with the virus containing the full-length *M. sexta* chitinase gene (Chi 1-535), as outlined in Section 2. Media (and cell extracts of AcMNPV–Chi 1-376 infection) recovered after 3–6 days of culture were analyzed by SDS-PAGE and proteins were stained with Coomassie Blue. Major protein bands with apparent sizes of 85, 66 or 55 kDa were detected in media from Chi 1-535-, Chi 1-477- or Chi 1-407, virus-infected cells, respectively

(Fig. 1, lanes, 2–4). Medium from AcMNPV lacking the *M. sexta* chitinase gene did not contain these prominent bands (lane 8). Western blot analysis with an antiserum to native *M. sexta* chitinase showed that the prominent 85, 66 and 55 kDa proteins were, indeed, immunologically related to *M. sexta* chitinase (data not shown). No immunologically reactive protein was detected in the medium from the AcMNPV-infected cells (data not shown). Therefore, the secretion of the Chi 1-407 and Chi 1-477 proteins was similar to the secretion of the full-length *M. sexta* chitinase. Removal of 58 amino acids (in Chi 1-477) and 128 amino acids (in Chi 1-407) from the C-terminal region of *M. sexta* chitinase, did not affect their synthesis or secretion by insect cells. Removal of the C-terminal 159 amino acids (in Chi 1-376), however, did affect secretion. When the medium from cells infected with the Chi 1-376 virus was analyzed no major protein band was seen in the Coomassie Blue-stained gel (lane 7) and no immunologically reactive protein was detected with the *M. sexta* chitinase antiserum (data not shown). However, an extract of proteins from the cells infected with this virus contained a prominent protein band(s) in the size range of 43–45 kDa (lane 6) compared to extracts from cells infected with AcMNPV lacking the *M. sexta* chitinase gene (lane 5). This broad band contained two closely spaced protein bands that cross-reacted with the *M. sexta* chitinase antibody (data not shown). Therefore, it can be concluded that deletion of the C-terminal amino acids of *M. sexta* chitinase up to residue 377 (but not up to 407 or 477) affected protein secretion and led to intracellular accumulation of this protein.

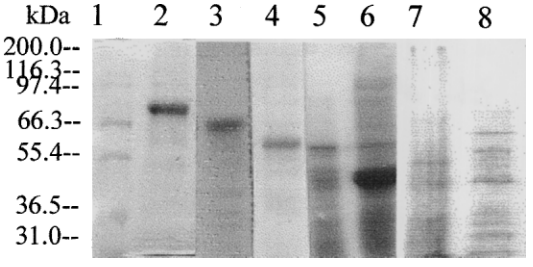

Fig. 1. Expression of Chi 1-376, Chi 1-407 and Chi 1-477 in a baculovirus-infected Hi-5 cell line protein expression system. Hi-5 cells were infected with the wild type AcMNPV or the recombinant baculoviruses containing the full-length chitinase gene or the three truncated forms of the gene as described in Section 2. Culture media (and cell lysate in the case of Chi 1-376) were collected after 3, 4 or 5 days of infection and aliquots (50 μl) were analyzed by SDS-PAGE followed by staining with Coomassie Blue. Lane 1, protein size standards; lane 2, culture medium collected 3 days after infection with Chi 1-535 virus; lane 3, culture medium collected 3 days after infection with Chi 1-477 virus; lane 4, culture medium collected 3 days after infection with Chi 1-407 virus; lane 5, extract of cells 3 days after infection with AcMNPV; lane 6, extract of cells 3 days after infection with the Chi 1-376 virus; lane 7, culture medium collected 3 days after infection with Chi 1-376 virus; lane 8, culture medium collected 3 days after infection with AcMNPV.

The Chi 1-407, Chi 1-477 and Chi 1-535 proteins secreted into the medium were purified to homogeneity by column chromatography as described in Section 2. The purified proteins were subjected to Edman degradation for the determination of N-terminal sequences. All three of the chitinases had the same N-terminal sequence, DSRARIV, which is the same as the N-terminal sequence of the enzyme isolated from molting fluid (Gopalakrishnan et al., 1995). Proteins extracted from the cell pellet fraction after infection with the Chi 1-376 virus also were subjected to SDS-PAGE in 12% polyacrylamide gels to resolve the two closely spaced bands that were present as major bands cross-reacting with the *M. sexta* chitinase antibody. These two bands were excised with as little contamination as possible from each other and subjected to N-terminal sequencing. The larger protein had the sequence, MRATLA, which corresponds to the N-terminal sequence of the signal peptide predicted from the *M. sexta* cDNA clone and the faster moving protein had the sequence, DSRAR, corresponding to the mature N-terminus. Thus, in the Chi 1-376 deletion mutant, signal peptide processing occurred but was incomplete. However, both processed and unprocessed forms were trapped inside the cell.

3.2. C-terminal truncation affects glycosylation

The molecular masses of the purified chitinases were determined by laser desorption mass spectrometry. The masses for the Chi 1-407 and Chi 1-477 were 49.4 and 60.0 kDa, respectively. These values were higher than the values predicted from the cDNA sequences of the truncated forms (after correcting for the removal of the leader peptide in the mature protein) by 3.3 and 6.5 kDa, respectively, and lower than the sizes estimated from their mobility on SDS-PAGE (55 and 66 kDa; Fig. 1). The full-length *M. sexta* chitinase is a glycoprotein with a carbohydrate content of approximately 25% (Gopalakrishnan et al., 1995). To investigate the possibility that the C-terminally truncated forms also may be glycosylated, carbohydrate composition analyses were carried out. Table 1 shows a comparison of the carbo-

Table 1
Carbohydrate content (mol/mol) of the three forms of *M. sexta* chitinases (moles of sugar per mole of protein)

Sugar	Chi 1-535	Chi 1-477	Chi 1-407
GalNAc	45.5	6.2	0.3
Mannose	15.5	8.9	5.5
Galactose	8.3	4.4	1.1
GlcNAc	7.7	2.3	0.6
Fucose	5.6	7.5	5.0
Glucose	2.0	3.2	3.1
Total[a]	84.6	32.5 (38%)[a]	15.6 (18%)[a]

[a] Percent relative to Chi 1-535 chitinase is given in parenthesis.

hydrate compositions of the three proteins. The two truncated forms have significantly lower amounts of total carbohydrate compared to the full-length form. The Chi 1-477 and Chi 1-407 proteins have about 38% and 18%, respectively, of the carbohydrate content of the full-length enzyme. These differences are due to the higher concentration of GalNAc, mannose, galactose and GlcNAc in the 85 kDa chitinase. The most dramatic difference is in the concentration of GalNAc followed by mannose, galactose and GlcNAc. The concentration of fucose was nearly the same in all three of the proteins.

3.3. Effect of C-terminal truncation on pH–activity profile

All three of the enzymes were active over a wide range of pH ranging from pH 5 to 10 (Fig. 2). The activity versus pH curves differed somewhat in the acidic range with the shorter proteins exhibiting more activity at pH 5.0, whereas the full-length form was more active in the neutral and alkaline pH range. The Chi 1-407 enzyme had the lowest specific activity, while the other two enzymes had nearly the same specific activity at pH values $\geq$6.

3.4. Temperature optima

Chi 1-407, Chi 1-477 and Chi 1-535 have a similar temperature dependence of their enzymatic activities (Fig. 3). The enzymatic activities for all three enzymes increased gradually as the temperature was raised from 0 to 45–50°C. The maximum activity was at 45°C for Chi 1-407, and at 50°C for both Chi 1-477 and Chi 1-535. The enzymatic activities of Chi 1-533 and Chi 1-477 started to decrease dramatically when the temperature was over 50°C. However, the enzymatic activity of Chi 1-407 fell markedly when the temperature was over 45°C, which indicated that Chi 1-477 and Chi 1-535 were slightly more stable than Chi 1-407.

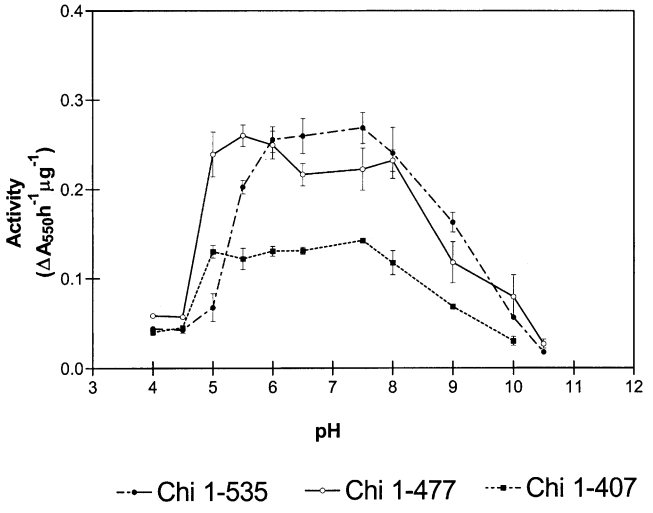

Fig. 2. Effect of pH on activities of Chi 1-407, Chi 1-477 and Chi 1-535. Enzyme activity was assayed using CM-Chitin-RBV as the substrate as described in Section 2. Data are presented as ΔA_{550}/h/μg enzyme. Mean±1/2 range (n=2).

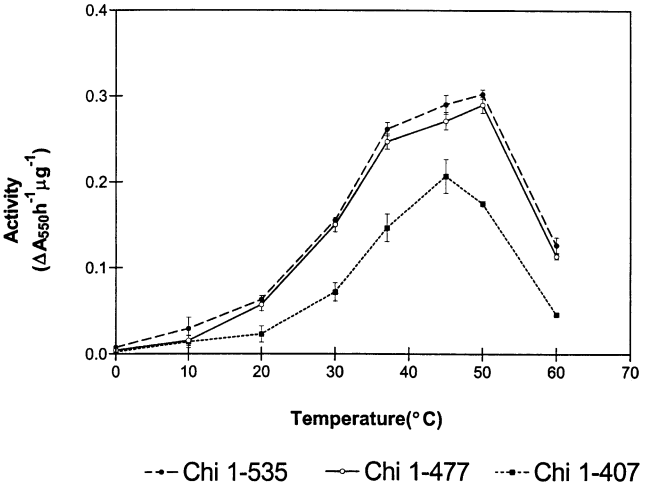

Fig. 3. Effect of temperature on activities of Chi 1-407, Chi 1-477 and Chi 1-535. Enzyme activity was assayed using CM-Chitin-RBV as substrate as described in Section 2. Data are presented as ΔA_{550}/h/μg enzyme. Mean±S.D. (n=3).

3.5. Influence of C-terminal truncation on kinetic parameters of the enzymes

The kinetic parameters of the three forms of *M. sexta* chitinase were determined from enzyme assays using either the fluorogenic oligosaccharide substrate, MU-$(GlcNAc)_3$, or the chromogenic polymeric substrate, CM-Chitin-RBV (Table 2). With the oligosaccharide substrate, all of the enzymes were very susceptible to substrate inhibition. A three-parameter substrate-inhibition model was used to calculate the K_m, K_i, and k_{cat} values with this substrate. With the polymeric substrate, the K_m and k_{cat} values were calculated using steady state

Table 2
Kinetic parameters of three forms of *M. sexta* chitinases

	Chi 1-535	Chi 1-477	Chi 1-407
A. Substrate=MU-$(GlcNAc)_3$			
K_m (μM)	2783	1738	4220
K_i (μM)	12.4	17.3	1.6
k_{cat} (s^{-1})	126.1	65.5	71.9
k_{cat}/K_m (M^{-1} s^{-1})×10^4	5	4	2
B. Substrate=CM-Chitin-RBV			
K_m (mg ml^{-1})	0.31	0.21	0.38
V_{max} (ΔA_{550}/nmol protein/h)×10^3	2.2	1.1	0.6
V_{max}/K_m (ΔA_{550}/nmol/h/mg ml)	83	98	29

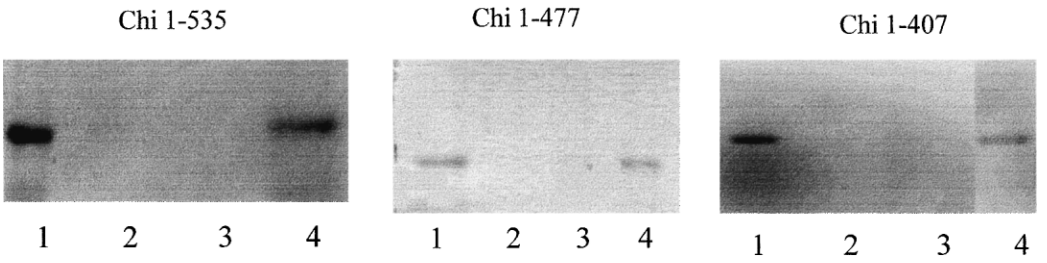

Fig. 4. Chitin-binding assay for Chi 1-407, Chi 1-477 and Chi 1-535. The binding assay mixture contained 100 µl (0.01 g) chitin, and 200 µl (3 µg protein) of each chitinase. The binding and elution of the chitinases from chitin were conducted as described in Section 2. All fractions were analyzed by SDS-PAGE followed by staining with Coomassie Blue. Lane 1, starting fraction; lane 2, unbound fraction; lane 3, wash fraction; lane 4, eluate fraction.

Michaelis–Menten kinetics because there was no evidence of significant inhibition even at relatively high substrate concentrations. With both substrates, the smallest K_m values were observed for the Chi 1-477 enzyme and the highest values were obtained with the Chi 1-407 enzyme. The V_{max} or k_{cat} values were the highest with the Chi 1-535 enzyme for both substrates and the lowest with the Chi 1-407 enzyme. The lowest V_{max}/K_m and k_{cat}/K_m ratios were exhibited by Chi 1-407.

3.6. Effect of truncations on chitin digestion and binding

To assess whether the removal of the C-terminal regions had any measurable effects on the affinity to chitin or the ability to digest chitin, two different types of experiments were carried out. The first one determined the ability of the chitinase to be retained on a chitin column (Fig. 4). Under the conditions of our chitin-binding assay, all three of the chitinases studied here retained their affinity to chitin. In the second assay the ability of the chitinases to digest colloidal chitin was assessed using a plate assay as outlined by Watanabe et al. (1990). Whereas the full-length chitinase resulted in clear "plaques" of a relatively small diameter indicating complete digestion, the two C-terminally truncated forms yielded larger "turbid" plaques characteristic of both incomplete digestion and a faster diffusion of the enzymes away from the site of application (Fig. 5). These semi-quantitative results indicated that there was only a decrease, and not a total loss in affinity of the deletion forms for colloidal chitin.

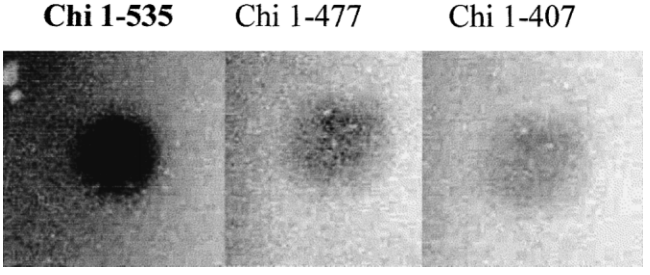

Fig. 5. Hydrolysis of colloidal chitin in agar by Chi 1-407, Chi 1-477 and Chi 1-535 enzymes. Each chitinase preparation (20 µg) was applied to a filter disk of 5 mm diameter and immediately placed on a colloidal chitin–agar plate as described in Section 2. The plate was incubated for 16 h at 37°C, stained with fluorescent brightener 28 and photographed under UV light.

4. Discussion

This work was prompted by the natural occurrence of C-terminal truncations of chitinolytic enzymes in several species including insects, nematodes, and bacteria. For example, the molting fluid of *M. sexta* has three chitinases with sizes of 85, 62 and 50 kDa (Koga et al., 1983). Because there is only one copy of the chitinase gene in the *Manduca* genome (Choi et al., 1997), these multiple chitinases are immunologically related and most likely are derived from the same mature protein by post-translational modifications. We also have observed the same three proteins when the *M. sexta* chitinase gene was expressed in insect cell lines during long culture (Gopalakrishnan et al., 1995). Similarly, *B. mori* molting fluid has three chitinases of sizes 88, 65 and 54 kDa, respectively, which have identical N-terminal sequences (Koga et al., 1997). The chitinase A2 of *Bacillus circulans* was shown to be derived from the larger A1 chitinase by proteolysis of a C-terminal fragment (Watanabe et al., 1990). The chitinase from the nematode, *Brugia malayi*, also shows susceptibility to proteolysis to yield an enzymatically active C-terminally truncated form of the enzyme (Venegas et al., 1996). Differences in enzymatic and chitin-binding properties were observed for some of these truncated forms. Additionally, removal of the C-terminal sequences from yeast (Kuranda and Robbins, 1991), *Brugia malayi* (Venegas et al., 1996), and *Clostridium paraputrificum* (Morimoto et al., 1997) chitinases was shown to result in loss of affinity for chitin. The naturally occurring 52 kDa chitinase from a parasitic wasp, *Chelonus* sp. near *curvimaculatus*, was found to have a serine/threonine-rich domain but lacked the C-terminal cysteine-rich domain found in other insect chitinases. Interestingly, this protein was not susceptible to inhibition by the oligosaccharide substrate unlike several other bacterial and insect chitinases. In this study, we created specific deletions that removed either the C-ter-

minal cysteine-rich region alone or the cysteine-rich region plus the serine/threonine-rich region to study how the loss of these regions affected the catalytic or chitin-binding properties of the *M. sexta* chitinase.

The presence of the N-terminal leader peptide coding region in the two deletion constructs Chi 1-407 and Chi 1-477 was apparently adequate to ensure correct and complete processing of the leader peptide and secretion of the corresponding enzymes into the culture medium as was observed with the construct coding for the full-length enzyme, because truncated forms of chitinases accumulated in the culture medium. The N-terminal sequence of all three of the chitinases was the same and there was no evidence of proteins with the leader peptide in the medium. On the other hand, the chitinase with the longest C-terminal deletion, Chi 1-376, was not secreted but accumulated inside the cell. We have not determined its exact intracellular location. One of the two immunoreactive forms produced by this expression construct had an N-terminus identical to the mature N-terminus of the *M. sexta* chitinase, whereas the other form has the N-terminus predicted for the unprocessed protein, suggesting that at least a part of the protein has been translocated into the endoplasmic reticulum (ER). It is possible that the absence of the sequences beyond position 376 may have an influence on the maturation (and/or glycosylation) of the protein during its transit from the ER to secretory vesicles. Because the larger proteins are secreted normally, the region between amino acid residues 377 and 407 may have a critical role in this process by affecting the glycosylation of the chitinase or by some unknown mechanism. Interestingly, the C-terminally truncated *M. sexta* chitinase expressed in transgenic tobacco (presumably truncated up to position 407) was not glycosylated and was in an intracellular location (Wang et al., 1996). This protein had the same N-terminal amino acid sequence as the *M. sexta* chitinase proteins reported in this study. The extracellular location of the Chi 1-407 enzyme expressed in the insect cell system suggests differences in the processing of this enzyme in plant and insect cells after entry/passage through the endoplasmic reticulum.

The C-terminal deletions also affected the nature and extent of glycosylation. Removal of only the last 58 amino acids at the C-terminus of *M. sexta* chitinase results in loss of nearly two thirds of the carbohydrate. Removal of an additional 70 amino acids results in the loss of 82% of the total carbohydrate from this protein, indicating that the major site of glycosylation is in the C-terminal region, which includes the serine/threonine-rich and cysteine-rich domains. The full-length 85 kDa chitinase has considerably greater amounts of galactosamine and galactose than the other two shorter forms. These sugars are lost nearly completely in Chi 1-407 and partially in Chi 1-477. Even though GalNAc is known to be present in terminal positions of some insect N-linked glycoproteins (Kubelka et al., 1995), galactose is not usually found in N-glycans. Furthermore, the ratio of galactosamine to mannose is much higher than would be expected from high mannose N-glycans with GalNAc. Galactose and galactosamine are typically found as O-glycans attached to serine and threonine. We had expected that the Chi 1-477 protein would retain almost all of the O-glycosylation because the serine/threonine-rich region is retained in this protein. In the region that is deleted from this protein, there are only five serine/threonine residues. Either these serine/threonines are the sites of major glycosylation or the removal of the C-terminal residues affects the structure of the protein in such a way as to prevent glycosylation of residues further downstream (for example, in the serine/threonine-rich region).

The loss of GlcNAc residues in the two deletions suggests the loss of N-glycosylation sites. There is only one glycosylation site (NAT, amino acid position 526–528) in the C-terminal region of *M. sexta* chitinase deleted in Chi 1-407 and Chi 1-477. Thus, the loss of about 5 mol of GlcNAc per mole of protein in Chi 1-477 and an additional loss of about 2 mol of this sugar in Chi 1-407 is a puzzle that remains unresolved. Analysis of the structure of carbohydrate chains released by N-glycosidase digestion may resolve this issue. Complex N-glycans with mannose, GlcNAc and GalNAc have been reported in the outer branches of N-glycans in honey bee hyaluronidase (Kubelka et al., 1995). The reduction in mannose content in the two deletions is consistent with the loss of one or two high-mannose N-glycan chains. There are three other N-glycosylation sites in this enzyme besides the one close to the C-terminus. There is very little change in the amount of fucose in the two deletions (the apparent increase in Chi 1-477 may be due to some contamination in that sample), indicating retention of at least one glycosylation site containing a complex oligosaccharide in these two enzymes.

Chi 1-477 and Chi 1-535 enzymes are slightly more active and more thermostable than Chi 1-407. The Chi 1-407 protein had the lowest specific activity and V_{max}/K_m or k_{cat}/K_m ratios compared to the two larger forms. This finding is consistent with the previous report that a truncated form of the *M. sexta* chitinase isolated from transgenic tobacco plants had only a fourth of the specific activity of the full-length enzyme isolated from the molting fluid (Wang et al., 1996). This enzyme had a lower V_{max} or k_{cat} than the full-length enzyme. It is clear that C-terminal deletions larger than the Chi 1-477 deletion adversely affect the catalytic activity of the enzyme. All three of the purified enzymes exhibited substrate inhibition behavior with the oligosaccharide substrate, MU-$(GlcNAc)_3$, indicating that these enzymes are normally not active on oligosaccharide substrates and prefer longer substrates. The Chi 1-407 had the lowest K_i value, however, indicating that the removal of the

serine/threonine-rich region resulted in greater susceptibility to inhibition by this substrate. This result was unexpected, because the chitinase from wasp venom, which has the serine/threonine-rich region but lacks the cysteine-rich region, apparently was not inhibited by this substrate (Krishnan et al., 1994). It is possible that there are differences in the substrate binding clefts of these enzymes, which account for the differences in inhibition by oligosaccharides. It is worth noting that chito-oligosaccharides are the products of chitinase action and the observed inhibition by MU-$(GlcNAc)_3$ may be a manifestation of product inhibition rather than substrate inhibition in vivo.

The C-terminal cysteine-rich region in insect chitinases has been postulated to be involved in chitin binding (Kramer and Muthukrishnan, 1997; Shen and Jacobs-Lorena, 1999; Coutinho and Henrissat, 1999). The binding of *M. sexta* chitinase to regenerated chitin is apparently less tight compared to the yeast or nematode enzymes (Kuranda and Robbins, 1991; Venegas et al., 1996). For example, the yeast enzyme binds to chitin in the presence of 5 M NaCl, whereas the *M. sexta* enzyme does not bind to chitin even in 0.1 M NaCl (unpublished data). In our standard chitin-binding assay in the absence of NaCl, all three of the enzymes studied here bound to chitin suggesting that the C-terminal region is not essential for the observed weak chitin binding. A similar finding has been reported for the C2 chitinase of *Serratia marcescens*, which lacks the C-terminal chitin-binding domain but retains significant chitin-binding activity (Suzuki et al., 1999). However, the C-terminal cysteine-rich region does have a role in chitin binding as shown in the enzymatic assay with colloidal chitin in agar. In this assay, the greater affinity for chitin of the full-length *M. sexta* chitinase compared to the truncated forms of chitinase could be demonstrated by the appearance of smaller zones of exhaustive hydrolysis. The C-terminally truncated forms produced zones of incomplete digestion because they were apparently more free to diffuse as a result of their lower affinity for colloidal chitin. This is similar to the findings of Watanabe et al. (1994) for chitinase A1 and a C-terminal deletion of *B. circulans* chitinase. Suetake et al. (2000) have shown recently using NMR spectroscopy that tachycitin, a chitin-binding protein from horseshoe crab, has a C-terminal domain with a tertiary structure closely resembling that of hevein, a plant chitin-binding protein whose crystal structure and chitin-binding domain are known. The C-terminal domain of *M. sexta* chitinase (positions 478–535) proposed to bind to chitin has an amino acid sequence related to tachycitin. Thus, it appears likely that the cysteine-rich C-terminal domain of *M. sexta* chitinase also is involved in chitin binding. This conclusion is consistent with the finding that other proteins with related cysteine-rich domains, such as peritrophin 44 from *Lucilla cuprina* (Elvin et al., 1996), the mosquito peritrophic matrix protein Ag-APer1 (Shen and Jacobs-Lorena, 1998), *Trichoplusia ni* intestinal mucin (Wang and Granados, 1997), a peritrophin-like protein in *Drosophila melanogaster* (Barry et al., 1999), and a modular protease in *Anopheles gambiae* (Danielli et al., 2000) also possess affinity for chitin.

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